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Antibacterial and Antioxidant Effects of *Citrus limon-vitis vinifera* Leaf Combination

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A B S T R A C T

The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries and for the presence of antimicrobials, anticancer agents and antioxidants. In the present study *Citrus limon* together with *Vitis vinifera* leaves were selected to evaluate their combined antibacterial and antioxidant properties. Different extractions using water, ethanol, methanol of *Citrus limon* and *Vitis vinifera* leaves combination were prepared to assess their antimicrobial and antioxidant properties. Antimicrobial testing by disc diffusion assay exhibited broad spectrum of antimicrobial activity for ethanol and methanol extracts of *C. limon* and *V. vinifera* leaves combination against various Gram-positive and gram-negative bacteria. *E. coli* and Pseudomonas sp. were observed to be most sensitive against the ethanol and methanol extracts. The aqueous extract of leaves showed less antimicrobial activity. In-vitro antioxidant potential of ethanol, methanol and aqueous extracts of *C. limon* and *V. vinifera* leaves combination using DPPH, FRAP, Phosphomolybdenum assay, Metal chelating assay, Hydroxyl radical scavenging activity, H_2O_2 scavenging method, showed significant percentage of inhibition in a dose dependent manner with Vitamin C and EDTA as a standard reducing agent. Ethanol and methanol extracts showed pronounced antioxidant activity than aqueous extract which could be attributed to the presence of alkaloids, flavonoids and saponins. It can be concluded that ethanol and methanol extracts of Citrus limon and Vitis vinifera leaves combination have potential antibiotic property against a large number of disease-causing bacteria and against various free radical initiated diseases.

***Key words*:** *Citrus limon, Vitis vinifera* leaves, Combination, Ethanol, Methanol, Aqueous extracts, Antimicrobial, Gram-positive and gram-negative bacteria, Antioxidant

Medicinal plants are a significant part of natural wealth. They serve as vital therapeutic agents as well as valuable raw materials for manufacturing numerous traditional and modern medicines [1]. Today a number of chemicals obtained from plants are used as vital drugs in more countries in the world [2]. Secondary metabolites from plants are referred to as phytochemicals which are naturally occurring and biologically active compounds that have the potential to prevent diseases. Evaluation of the phytochemical constituents of a medicinal plant is considered to be the main step in medicinal plant research [3]. Traditional medicine based on plants has played a key role in the health care system of many countries like India, China etc., [4]. About 60% of the total global population remains dependent on traditional medicines for their health care system [5].

Plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth [6] and have a great potential for producing new drugs for human benefit. Traditional medicine prepared using plants contain a vast array of substances that can be used to treat chronic and infectious diseases. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke [7]. The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark [8]. Drug combination in Ayurveda is very effective as they mostly found to have wide therapeutic applications.

Most of them are effective even at a low dose and safe at high dose. Based on this we chose two medical plants: *Citrus limon* whole fruit and *Vitis vinifera* leaves for our drug combination studies. Lemon is very rich in important natural compounds, including citric acid, ascorbic acid, minerals, flavonoids, and essential oils. Therefore, although the new *Citrus* cultivars have been mainly developed for fresh consumption, the particular characteristics such as their phenolic compound and in particular the flavonoids contents have led to their use in new fields such as pharmacology and food technology [9].

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Similarly leaves of *Vitis vinifera* (grapes) extract investigated by reversed phase HPLC showed the presence of resveratrol, viniferin, balanocarpol [10]. It was reported that the leaves are rich in tannin, flavonoid, procyanidin, organicacids, lipids, enzymes, vitamins [11]. The fresh leaves contain procyanidins, anthocyanins, flavanoids, hydroxyl cinnamic acid derivatives, triterpenes, sterols, tannins, polysaccharides, monosaccharides and non-alkaloid nitrogen containing compounds. Thus, it is thought that herbal remedies have the advantage in combining two or more plants to obtain synergistic or additive effects to yield better therapeutic potency [12]. The goal of the present study is to screen the phytochemicals and to compare the *invitro* antimicrobial, antioxidant potential of *Citrus limon* and *Vitis vinifera* leaves combination extracted using different solvents such as water, ethanol and methanol.

MATERIALS AND METHODS

1. *Collection, preparation of the sample*

Whole *Citrus limon* fruits were collected from the local market and dried in hot dry oven (50 °C). *Vitis vinifera* leaves were collected from nearby field (Cumbum, Theni Dt). The plant material was air dried in the shade at room temperature. The dried plant material was powdered using mixer grinder.

2. *Extraction and sample preparation*

i) *Aqueous extraction*

For aqueous extraction, 10 g of air-dried powder of *Citrus limon* and 10 g of *Vitis vinifera* leaves together was placed in 100 mL distilled water and boiled for 5 hrs. Then it was filtered through muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected and crude aqueous-leaf extract was obtained. Then it was transferred in to screw cap bottles, labeled and stored under refrigerated (4 °C) condition until use.

ii) *Solvent extraction*

10g of *Citrus limon* powder and 10g *Vitis vinifera* leaf powder were weighed, mixed together and extracted with 95% ethanol, 95% methanol and water separately. The crude preparation was left for 72 hours in shaker at room temperature. The extract obtained by cold extraction was then concentrated using vacuum evaporator. A greasy final material (crude ethanolic combination extract, crude methanolic combination extract) obtained was transferred to screw cap bottles, labeled and stored under refrigerated (4 °C) condition till use.

3. *Antimicrobial assay*

Microbial strains

Pure cultures of bacteria namely *Escherichia coli, Micrococcus sp., Bacillus sp., Pseudomonas aeruginosa, Streptococcus sp., staphylococcus aureus, Shigella sp., Vibrio sp., Klebsiella pneumonia* and *Neisseria sp.* were obtained from Department of Microbiology, V. V. Vanniaperumal College for Women, Virudhunagar. Overnight cultures of the above-mentioned strains were used for this study.

Agar well diffusion method

The agar well diffusion method was employed for the determination of antimicrobial activity. Wells were made in the ager plate with a sterile cork borer and the inoculums containing 50 µl of microbial strains was spread on the plates with the help of glass spreader in an aseptic condition. 25µl/ml extracts of *Citrus limon* and *Vitis vinifera* leaves combination was filled in wells with the help of sterile micropipettes separately. Ampicillin was used as the reference standard. The aqueous, ethanolic, methanolic test extracts were individually tested against test organisms. The plates were incubated at 37 °C for24 hours. The diameter for the zone of inhibition was measured in millimeter (mm). For each extract, three replicates were maintained. Each zone of inhibition was measured with a ruler and compared with the control [13].

4. *Antioxidant activity*

The antioxidant activity of ethanol, methanol and aqueous extracts of *Citrus limon* and *Vitis vinifera* leaves were measured DPPH assay, Ferric (Fe^{3+}) reducing power assay, Phosphomolybdenum reduction assay, Metal Chelating Assay, Hydroxyl Radical Scavenging Activity Assay and by Scavenging of Hydrogen peroxide activity.

RESULTS AND DISCUSSION

Plants have provided a source of inspiration for novel drug compounds as plant derived medicines have made significant contribution towards human health. The phytochemical constituents are responsible for the biological and pharmacological actions of these plants. Traditional healers and local tribal people are generally used water as the solvent. Most of the phytochemicals are extracted by using organic solvents such as ethanol, methanol, ethyl acetate etc. Further trials using solvents of various polarities will explore the effects of solvent composition on extract efficacy [14]. Moreover combinational effect of two plant sources has great potentiality of imparting therapeutic outcomes such as anti -microbial, anti-diabetic, anti- cancer effects by stimulating the anti-oxidant property. The observed results further support the view that some traditionally used Indian medicinal plants are promising sources of potential antioxidants and medicinal compounds. The study is also aimed to compare the antioxidant activities of different solvent extracts. Phytochemical constituents dissolve in polar solvents with maximum efficiency thus exhibiting their potent anti-microbial and anti -oxidant activities.

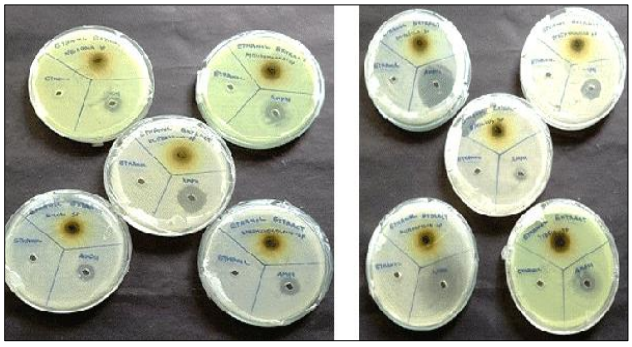

Fig 1 Antimicrobial activity of combined *C. limon* and *V. vinifera* leaves ethanol extract against gram positive and gram-negative bacteria

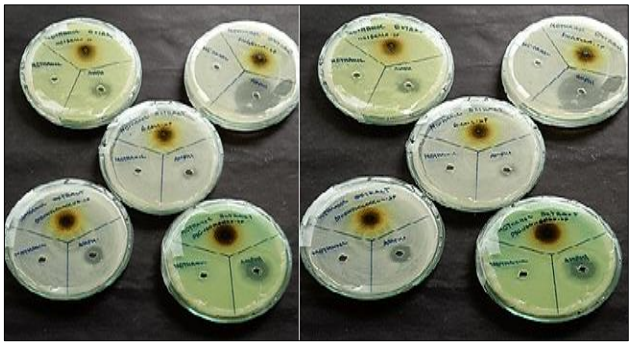

Fig 2 Antimicrobial activity of combined *C. limon* and *V. vinifera* leaves methanol extract against gram positive and gram-negative bacteria

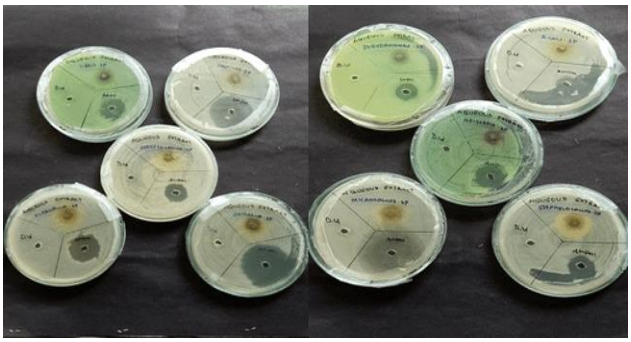

Fig 3 Antimicrobial activity of combined *C. limon* and *V. vinifera* leaves aqueous extract against Gram-positive and Gram-negative bacteria

Plants have been valuable and indispensable sources of natural products for the health of human beings and they have a great potential for producing new drugs [15]. Bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [16]. The pharmacological industries have produced a number of new antibiotics; resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to synthetic drugs which are utilized as therapeutic agents [17]. The results of antimicrobial susceptibility assay showed promising evidence for the antimicrobial effects of ethanol and methanol extract of the *Citrus limon* and *Vitis vinifera* leaves combination against bacterial pathogens.

Table 1 Antimicrobial activity for ethanol extract of *C. limon* and *V. vinifera* leaves combination (Zones measured in mm)

Microorganisms	Ethanol extract	Ampicillin	P values
E. coli	12.67 ± 0.088	14.33 ± 0.129	0.3262
Bacillus	17.33 ± 0.296	21.33 ± 0.32	0.4169
Pseudomonas	19.33 ± 0.233	16.67 ± 0.406	0.5992
Micrococcus*	14.33 ± 0.120	25.00 ± 0.289	0.0270
Streptococcus*	12.33 ± 0.033	16.33 ± 0.088	0.0132
Staphylococcus**	11.67 ± 0.067	16.00 ± 0.058	0.0080
Shigella	12.00 ± 0.058	22.00 ± 0.656	0.2034
Vibrio	16.67 ± 0.176	18.67 ± 0.371	0.6520
Klebsiella	16.67 ± 0.120	22.00 ± 0.462	0.3264
Neisseria	13.67 ± 0.088	14.00 ± 0.153	0.8593

Data are means of three replicates (n=3) ±SEM,
*Significant at two tailed P=0.0004 compared to standard
**Significant at two tailed P=0.0001 compared to standard

Table 2 Antimicrobial activity for methanol extract of *C. limon* and *V. vinifera* leaves combination (Zones measured in mm)

Microorganisms	Ethanol extract	Ampicillin	P values
E. coli	11.67 ± 0.033	14.67 ± 0.088	0.00335
Bacillus	17.69 ± 0.318	20.33 ± 0.318	0.5851
Pseudomonas	19.33 ± 0.333	21.00 ± 0.346	0.7463
Micrococcus*	14.33 ± 0.067	22.33 ± 0.283	0.0522
Streptococcus*	14.33 ± 0.133	17.33 ± 0.133	0.1868
Staphylococcus**	13.00 ± 0.100	15.33 ± 0.145	0.2564
Shigella	12.00 ± 0.058	20.67 ± 0.570	0.2046
Vibrio	18.33 ± 0.353	20.00 ± 0.306	0.7390
Klebsiella	15.67 ± 0.033	24.00 ± 0.404	0.1091
Neisseria	13.33 ± 0.120	16.00 ± 0.208	0.3295

Data are means of three replicates (n=3) ±SEM,
*Significant at two tailed P=0.0004 compared to standard
**Significant at two tailed P=0.0001 compared to standard

Different extracts of *C. limon* and *V. vinifera* leaves combination were screened for their antimicrobial activity against selected bacterial strains (*E. coli*, Staphylococcus aureus, Klebsiella pneumoniae, Bacillus, pseudomonas aeruginosa, Micrococcus, Streptococcus, Shigella, Vibrio, Neisseria). The data presents the antimicrobial activity of the ethanol and methanol extracts of *C. limon* and *V. vinifera* leaves combination (Table 1-2). Ethanol extract exhibited similar zone of inhibition as compared to the standard antibiotic (ampicillin) in *E. coli*, Vibrio, Neisseria. Maximum inhibition was observed in Pseudomonas sp. Methanol extract exhibited its maximum antimicrobial potential against Pseudomonas. It also exhibited similar zone of inhibition as compared to ampicillin in *E. coli*, *Bacillus*, Streptocococcus, Staphylococcus, Neisseria. Maximum zone of inhibition for both ethanol and methanol extract was observed against *Pseudomonas sp*. in the range of 19.3 mm. Both ethanol and methanol extract exhibited the zone of inhibition above 10 mm for all the tested organisms which brings out the potent antimicrobial activity of *C. limon* and *V. vinifera* leaves combination extracted using ethanol and methanol (Fig 1-2). The antimicrobial activity of the *C. limon* shows maximum inhibition against *E. coli* and *Bacillus* in the range of 10mm and 9mm in diameter [18], but our study showed 12.67mm and 17.33mm for ethanol extract and 11.67mm and 17.69mm for methanol extract because of the combination with *V. vinifera* leaves. So, the combination effect shows more inhibition against microorganisms compare to the *C. limon* alone. At the same time aqueous extract exhibited no zone of inhibition in all the tested microorganisms. It might be due the absence of secondary metabolites in the extract (Fig 3).

Different reports from different countries using different pathogens were published exhibiting the antimicrobial activities of medicinal plants [19]. Many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites [20]. Mahesh and Satish [21] studied the methanolic leaf extracts of *Acacia nilotica*, *Sida cordifolia*, *Tino spora cordifolia*, *Withania somnifer* and *Ziziphus mauritiana*. They

reported antibacterial activity against clinical pathogen *Escherichia coli*. Ahmad *et al*. [22] investigated the antibacterial activity of *Vitis vinifera* leaf extracts against some pathogenic bacterial strains. Sample consisting of fresh healthy leaf that were tested for their properties to inhibit growth of four species of bacteria, namely, *Staphylococcus aureus Pseudomonas aeruginosa*, *Enterococcus faecalis* and *E. coli*.

Antioxidant activities

Complex antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species. Several medicinal plants (Rasayana) have also been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of number of diseases [23]. It is known fact that antioxidants are required to suppress the free radicals like reactive oxygen species are generated in the body. Reactive oxygen species from both endogenous and exogenous source may be involved in the etiologies of such diverse human diseases as arteriosclerosis, cancer and neurodegenerative diseases, as well as in the processes like inflammation and ageing. The antioxidant activity cannot be evaluated by only a single method due to the complex nature of phytochemicals. Also, the antioxidant activity determination is reaction-mechanism dependent. Therefore, it is important to employ multiple assays to evaluate the antioxidant activity of plant extract or phytochemicals [24]. Determination of the natural antioxidant compounds of plant extracts will help to develop new drug candidates for antioxidant therapy [25]. The plants may be considered as a good source of natural antioxidants for medicinal uses such as against aging and other diseases related to radical mechanisms [26].

a. *DPPH radical scavenging assay*

The electron donation ability of natural products can be measured by DPPH radical purple coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. Ethanol extract of *C. limon* and *V. vinifera* leaves combination indicates higher antioxidant activity as same as that of standard ascorbic acid. The methanol extract exhibited remarkable antioxidant activity but little lower than that ethanol extract (Fig 4). The antioxidant activity of aqueous extract was not so remarkable as it exhibited lesser percentage of inhibition as compared with standard. DPPH is a stable free radical, which has been widely used in phytomedicine for the assessment of scavenging activities of bioactive fractions. Thus, from the results it is very evident that ethanol and methanol extract have shown very good percentage of DPPH assay and thus showing a great potential for future research and use in medicinal field and treating variety of diseases. Several studies demonstrated a linear correlation between antioxidant activity and phenolic content of plant extracts [27]. Our findings were not an exceptional case, as they indicated that *Citrus limon* and *Vitis vinifera* leaves extract consists high quantity of phenolic compounds and showed promising antioxidant activity.

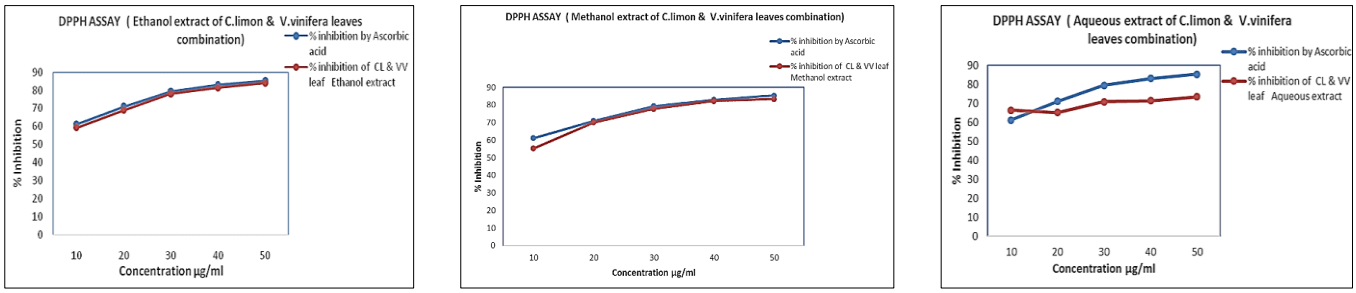

Fig 4 DPPH assay (Ethanol, Methanol, aqueous extracts of *C. limon and V. vinifera* leaves combination)

b. *Ferric reducing assay power*

The Ferric Reducing Assay Power (FRAP) value measures the reduction of the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) by donor electrons in the sample [28]. The most active extract in the FRAP assay was the ethanol extract followed by methanol extract (Fig 5). Aqueous extract exhibited least measure of reducing ferric ions. In summary, extracts with excellent electron donating ability were the ethanol extract. Electron donating ability was good for the methanol extract and least for aqueous extract. The ferric reducing/antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant component in dietary polyphenols [29]. Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species [30].

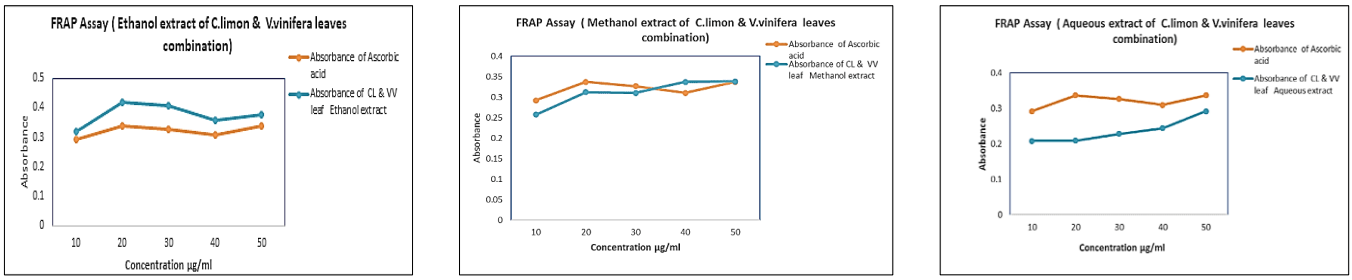

Fig 5 FRAP assay (Ethanol, Methanol and aqueous extract of *C. limon* and *V. vinifera* leaves combination)

c. *Phosphomolybdenum assay*

The total antioxidant capacity of the fractions was determined by phosphomolybdate method using ascorbic acid as a standard [31]. The phosphomolybdate method is quantitative, since the total antioxidant capacity (TAC) is expressed as ascorbic acid equivalents. The antioxidant capacity of various solvent fractions of *Citrus limon* and *Vitis vinifera* leaves combination was found to decrease in this order:

Methanol extract > ethanol extract > Aqueous extract (Fig 6). Strong antioxidant activity of methanol statistically similar to ascorbic acid indicates strong antioxidants in this fraction and these could be attributable to the presence of phenolic compounds (Fig 19). Similar finding by Fidrianny *et al*. [32] in *Citrus maxima* reported the effect of various extracts and their effective antioxidant ability.

d. *Metal chelating assay*

The chelating effect on the ferrous ions by ethanol, methanol and aqueous extracts of *Citrus limon* and *Vitis vinifera* leaves combination are presented in (Fig 7). The methanol extract exhibited the greater ability to chelate metal ions similar to that of EDTA. Comparatively ethanol extract also showed metal chelating effect in line with EDTA. Aqueous extract also was in close line with EDTA revealing that it is not devoid of antioxidants. Chelation of iron plays the main role for assessing antioxidant potential of medicinal plants. Evidence state that ROS formation is accelerated by metals such as iron and copper. The origination of ROS associated with redox-active metal catalysis could be circumvented by chelating the metal ions [33]. Antioxidants in the extracts forms an integrated complex with the metal ions that best describes its chelating activity and hinders the electron transfer. Thus, oxidation of cellular metabolic reaction is seized, resulting in the absence of free radicals production.

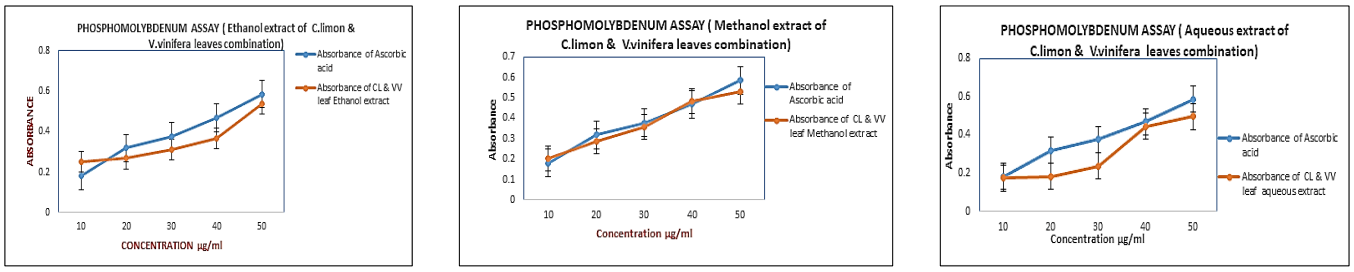

Fig 6 Phosphomolybdenum assay (Ethanol, Methanol and aqueous extract of *C. limon* and *V. vinifera* leaves combination)

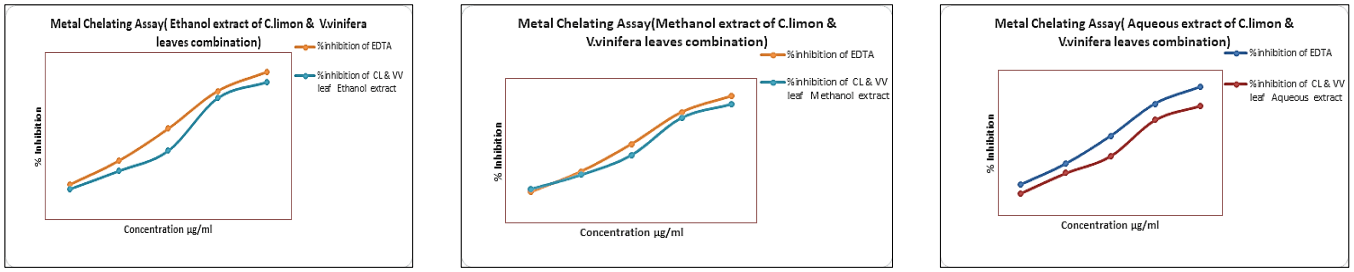

Fig 7 Metal chelating assay (Ethanol, Methanol and aqueous extract of *C. limon* and *V. vinifera* leaves combination)

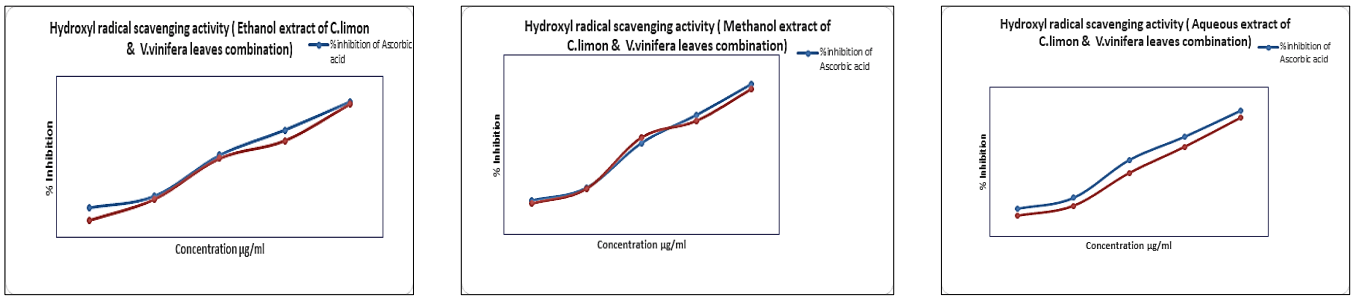

Fig 8 Hydroxyl radical scavenging activity (Ethanol, Methanol and aqueous extract of *C. limon* and *V. vinifera* leaves combination)

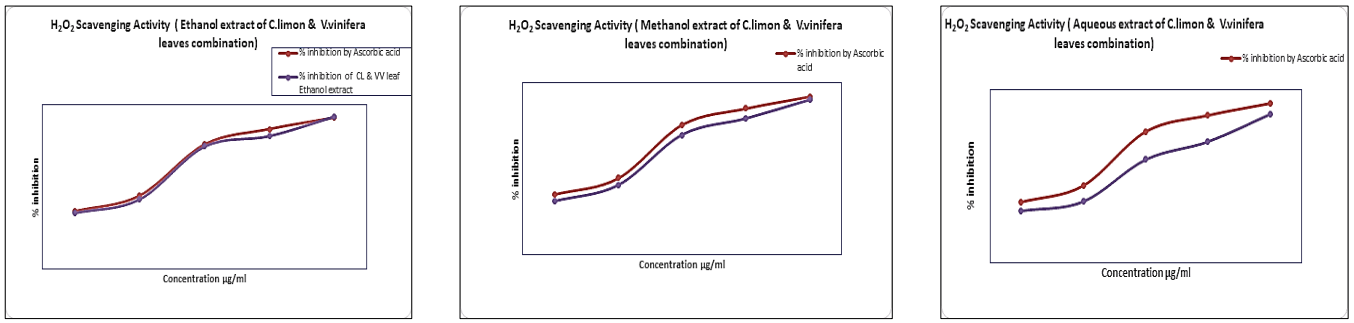

Fig 9 H_2O_2 scavenging activity (ethanol, methanol and aqueous extract of *C. limon* and *V. vinifera* leaves combination)

e. *Hydroxyl radical scavenging activity*

Hydroxyl radical inhibition of 3 extracts of *Citrus limon* and *Vitis vinifera* leaves combination were investigated and these results are shown as relative activity against the standard (Vitamin C). Hydroxyl radical scavenging activity of CB AgNPs is presented in (Fig 8). There is no significant difference in the hydroxyl radical scavenging activities of the ethanol and methanol extracts revealing that they are potent in scavenging hydroxyl radicals similar to that of vitamin C. The aqueous extract exhibited less activity as compared to ethanol and

methanol extracts. Hydroxyl radical is an extremely reactive free radical formed in biological system and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule, proteins, DNA (Deoxyribonucleic acid), unsaturated fatty acids and lipids in almost every biological membranes found in living cells [34-35]. Our results are consistent with the findings of Shan *et al*. [36] which reported the hydroxyl scavenging activity of different extracts of *Duchesnea indica, Prunus domestica* and *Rubus ellipticus.*

f. *H_2O_2 scavenging activity*

The data of H_2O_2 scavenging activity suggests that the ethanol and methanol extracts were more potent in scavenging hydro free radicals and was much comparable with the standard Ascorbic acid (Fig 9). Aqueous extract also holds H_2O_2 scavenging activity but much lesser than that of standard.

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells [37]. Thus, the removing of H_2O_2 is very important for antioxidant defense in cell or food systems. Hydrogen peroxide inactivates a few enzymes directly, usually by oxidation of essential thiol group (-SH). responsible for various toxic effects can cross membranes and reacts with Fe^{2+} and Cu^{2+} ions to form hydroxy radical. H_2O_2 scavenging activity of different extracts of *Citrus assamensis* leaves was reported which resembled our results [38].

CONCLUSION

Overall, by this study it can be concluded that ethanol and methanol extracts of *Citrus limon* and *Vitis vinifera* leaves combination have potential antibiotic property. They are effective against a large number of disease-causing bacteria. The phytochemicals present in these extracts magnified their antioxidant efficacy which is necessary in suppressing free radicals that are generated in our body during unfavorable conditions. Moreover, the phytochemical fractions may be purified further to isolate the single active component from the fractions and its structure can be elucidated. The active component can be subjected to clinical trials to develop into a novel drug.

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